

# Taxonomical discrimination of pollen grains by using confocal laser scanning microscopy (CLSM) imaging of autofluorescence

A.J. Castro, J.D. Rejón, M. Fendri, M.J. Jiménez-Quesada, A. Zafra, J.C. Jiménez-López, M.I. Rodríguez-García and J.D. Alché,

Department of Biochemistry, Cellular and Molecular Biology of Plants, Estación Experimental del Zaidín (CSIC),  
Profesor Albareda 1, 18008 Granada, Spain

Several components of the exine (i.e. the outer layer of the pollen wall) show conspicuous autofluorescence in the pollen grain. We describe the autofluorescence properties of pollens from the Mediterranean cypress, lily, turnip and olive (cvs. Picual, Loaime, Cornicabra and Ayrouni). The intensity and pattern of autofluorescence largely varied depending on the species and the hydration stage of pollen. In the case of olive, differences in the level of autofluorescence were detected among the four cultivars analyzed. This technique also allowed further morphological characterization of pollen (e.g. determination of pollen size and shape, and the number and type of apertures, the pattern of the exine, etc), evaluation of pollen integrity, detection of additional components (e.g. the pollen coat) over the surface of the pollen wall, etc. The methods used can be considered non-disruptive and lack of sample preparation. Monitoring pollen autofluorescence could be useful in disciplines like Aerobiology, Plant Taxonomy, and Cell and Molecular Biology.

**Keywords** autofluorescence; CLSM; cultivars; exine; hydration; pollen; pollen coat; pollen wall; species; taxonomy

## 1. Introduction

Autofluorescence of living cells and tissues is an intrinsic property arising from the presence of endogenous molecules like aromatic amino acids, lipo-pigments and pyridine (NADPH) and flavin (FADH<sub>2</sub>) co-enzymes, which become fluorescent when excited by ultraviolet (UV) light [1, 2]. In plant cells, autofluorescence is mainly derived from the presence of chlorophyll in chloroplasts and lignin in secondary cell walls [3].

Similar to other plant cells and tissues, the pollen grain (i.e. the male gametophyte) also exhibits autofluorescence [4-6]. This property relies on compounds like carotenoids, phenolics (e.g. flavonoids) and terpenoids [7], which are firmly bound to the outer layer of the pollen wall (i.e. exine). The emission of autofluorescence has been used to assess pollen sensitivity to environmental factors such as ozone and peroxides [8-9], to study pollen retention in the stigma [10], to monitor pollen wall development [11-13] or simply to count pollen grains in an appropriate device [14-15]. Pollen autofluorescence levels change during its ontogeny and ageing. These changes have been correlated with pollen viability by several authors [16]. Autofluorescence has begun to be used for taxonomical discrimination. In a pioneer study, 15 out of 21 grass pollen species were classified on the basis of the autofluorescence colour of the cytoplasm and exine layers, and the overall autofluorescence intensity [17]. More recently, pollen from nine plant species were identified on the basis of its size and the ratio of the blue to red autofluorescence spectra [18].

In the present work, we examine the autofluorescence properties of mature pollen obtained from four plant species: olive, Mediterranean cypress, lily and turnip. In the case of olive, pollen from cultivars Picual, Loaime, Cornicabra and Ayrouni was imaged and analyzed.

## 2. Material and methods

### 2.1 Plant material

Mature pollen grains from olive (*Olea europaea* L., cvs. Picual, Loaime, Cornicabra and Ayrouni), Mediterranean cypress (*Cupressus sempervirens* L.), lily (*Lilium longiflorum* L.) and turnip (*Brassica rapa* L.) plants were sampled by vigorously shaking the flowering shoots inside paper bags. Olive, cypress and turnip pollen grains were collected from their natural habitats, whereas lily pollen was obtained from flowering shoots purchased in a local flower nursery. Pollen grains (approximately 0.05 g for each plant species) were hydrated by placing them on a wet filter paper and incubating at 30°C for 30 min in the dark.

### 2.2 CLSM imaging

The autofluorescence of pollen exine was imaged at different magnifications with a C1 confocal laser scanning microscope (Nikon, Japan) using a combination of He-Ne (543 nm) and He-Ne (633 nm) lasers. Autofluorescence emission was captured independently at 590-650 nm (orange-red spectrum) and 650LP nm (far red spectrum). In all cases, section stacks were generated, which were then projected into a single plane. Identical parameters for each

acquisition were used, in order to enable making comparisons of the registered autofluorescence. The image capture was performed using the EZ-C1 viewer v.3.30 software (Nikon, Japan). Pseudocolours were assigned to each type of pollen to differentiate species.

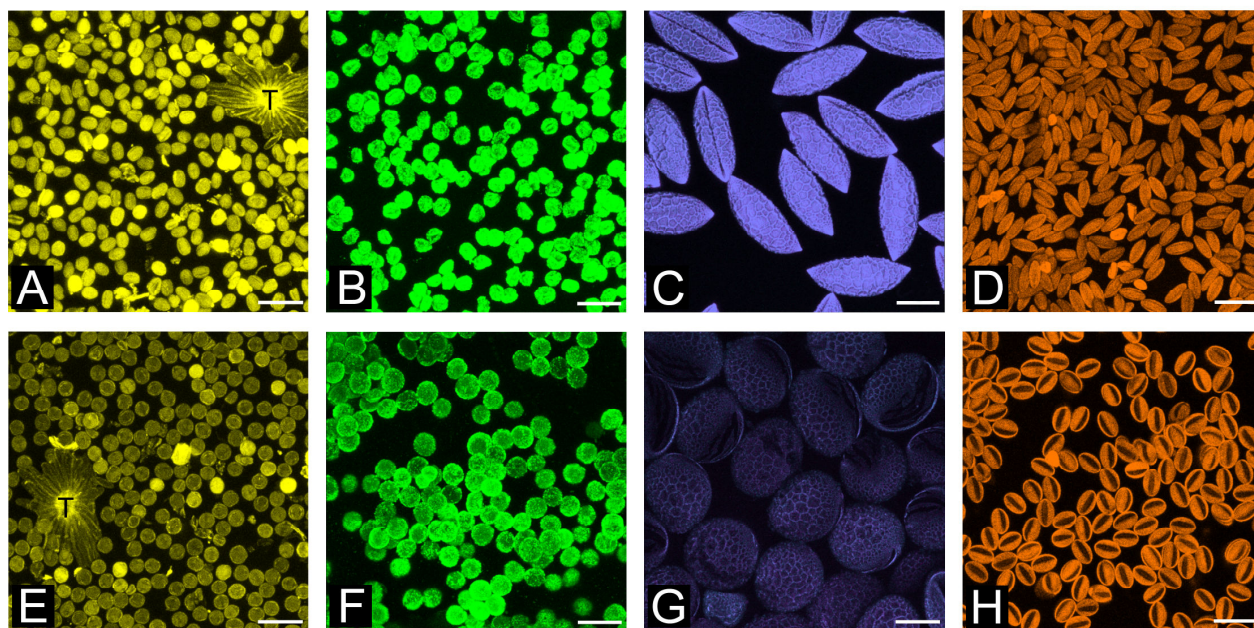
### 2.3 Measurement of pollen autofluorescence intensity and statistical analysis

The intensity of autofluorescence of intact pollen surfaces was calculated in arbitrary units (A.U.) per area unit using the EZ-C1 viewer v.3.30 software (Nikon, Japan). Means ( $N=20$  pollen grains per species)  $\pm$  SD were calculated and plotted using the SSPS Statistics v. 17.0.0 software (SSPS, USA). Non-parametric comparisons of variables (total autofluorescence intensity and 590/650 to 650LP fluorescence ratio) for two unpaired groups (Mann-Whitney test) and more than three unmatched groups (Kruskal-Wallis test) were performed using the SSPS Statistics v. 17.0.0 software (SSPS).

## 3. Results and Discussion

### 3.1 Discrimination of plant species by pollen autofluorescence

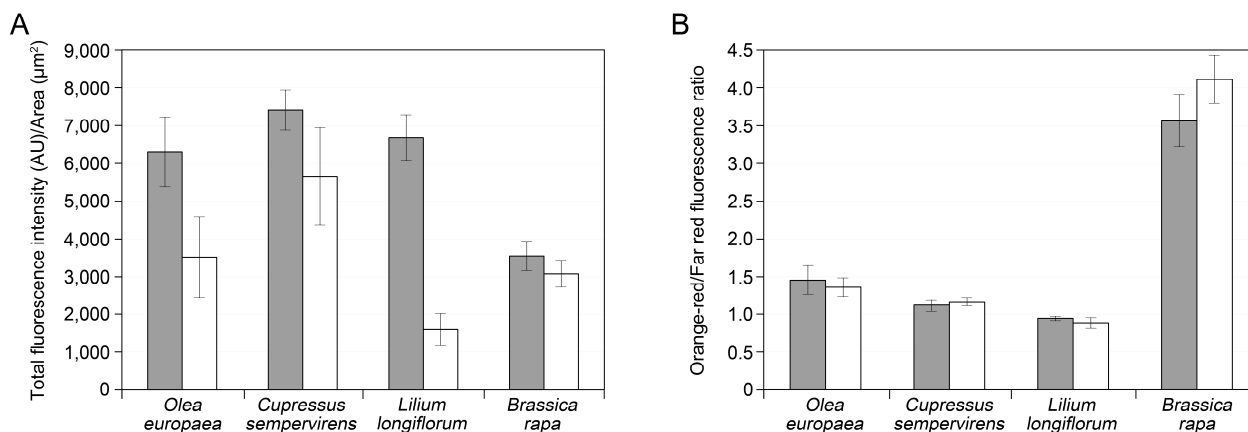
The autofluorescence intensity of the pollen varied depending on the species and its hydration status, as shown in Fig. 1. Contaminating structures (trichomes) in olive pollen samples also fluoresced but they were easily distinguishable from true pollen grains (Fig. 1A, E). Pollen autofluorescence mainly came from the exine wall, while the apertures did not fluoresce in any of the species analyzed (Fig. 1G, H). This fluorescence is likely due to carotenoids and phenols present in the exine, as described in other species [16, 19-22]. Indeed, the extraction of these compounds with organic solvents was demonstrated to lead to a loss of pollen autofluorescence [23]. We observed that the intensity of autofluorescence was heterogeneous in each pollen sample, some grains being brighter than others. This intraspecific variability was more marked in the olive and turnip and could reflect differences in the water content of each grain within a species (Fig.1).



**Fig. 1** Representative autofluorescence images of olive (cv. Picual), Mediterranean cypress, lily and turnip pollen before (Fig. 1A, B, C and D, respectively) and after (Fig. 1E, F, G and H, respectively) rehydration. Images were obtained with a confocal laser microscope and represent the sum of the fluorescence emitted at 590-650 nm and 650LP nm after excitation at 543 nm (green laser) and 633 nm (red laser), respectively. Different pseudocolours were used to differentiate species. T, trichome. Bars= 100  $\mu$ m.

The brightest autofluorescent signal was detected in the dry pollen, independently of the species. Then the autofluorescence intensity decreased as pollen becomes fully hydrated. It was previously described that water quenched the autofluorescence of viable pollen during *in vitro* germination [13] and upon contact of pollen with the stigma surface [20]. Supposedly non-viable pollen grains, however, remained fluorescent after pollen wetting. A similar pattern occurred in olive, cypress, lily and turnip. Figure 2 highlights the results of quantifying both the total fluorescence intensity and the green to red illumination-emitted fluorescence ratio for each type of pollen. The statistical analysis of these data showed significant differences in the total autofluorescence intensity among the species studied (Fig. 2A),

independently of whether the pollen grain was fully hydrated (Kruskal-Wallis test,  $\chi^2 = 55.664$ ,  $p = 0.000$ ) or not ( $\chi^2 = 49.996$ ,  $p = 0.000$ ). Before rehydration, cypress pollen produced the highest emission of autofluorescence, while turnip pollen showed the lowest values. These interspecific differences could be due to both quantitative and qualitative variations in the composition of the exine and/or differences in the degree of pollen hydration at maturity, as demonstrated in other plant species [9, 13]. Non-parametric comparisons of two unpaired groups of species revealed that the dry pollen grains of olive and lily present similar autofluorescence intensity values (Mann-Whitney test,  $U = 105.0$ ,  $p = 0.071$ ), but the rest of comparisons resulted in significant differences. After hydration, measurements indicated a different situation, with olive and turnip pollens showing similar values of autofluorescence ( $U = 130.0$ ,  $p = 0.311$ ), and the remaining comparisons resulting in significant differences.



**Fig. 2** Quantification of total autofluorescence intensity (A) and orange-red/far red autofluorescence ratio (B) of olive (*Olea europaea*), Mediterranean cypress (*Cupressus sempervirens*), lily (*Lilium longiflorum*) and turnip (*Brassica rapa*) pollen. Grey columns refer to dry pollen, whereas white columns refer to hydrated pollen. Means (N= 20)  $\pm$ SD are displayed for each plant species. AU= arbitrary units.

Pollen from olive, cypress and turnip showed a higher fluorescence emission in the 590-650 nm spectrum than in the far red (650LP nm) region (Fig. 2B). Contrary, lily pollen displayed higher autofluorescence values in the far red (650LP nm) emission wavelength. Interestingly, the orange-red/far red fluorescence ratio also varied significantly among the species studied (Fig. 2B) in both hydrated (Kruskal-Wallis test,  $\chi^2 = 64.680$ ,  $p = 0.000$ ) and dry ( $\chi^2 = 63.884$ ,  $p = 0.000$ ) pollen. Non-parametric comparisons of G/R fluorescence for two unpaired groups of species always resulted in significant differences, independently of the water status of pollen. Therefore, this last parameter alone or in combination with the autofluorescence intensity allowed classifying the four plant species studied.

Autofluorescence visualization also allowed identifying additional pollen morphological traits (Table 1). Parameters like pollen shape and size (i.e. pollen diameters), exine pattern, and type and number of pollen apertures were easily determined in our samples (Fig. 3A-B). Thus, lily pollen was almost four-fold larger in diameter than the other species studied, and experienced a drastic change in its shape after hydration. Turnip pollen kept its oval shape after hydration while pollen grains rounded off in the other species. All together, these parameters might be a valuable supplement to the autofluorescence data for fine discrimination of a higher number of plant species.

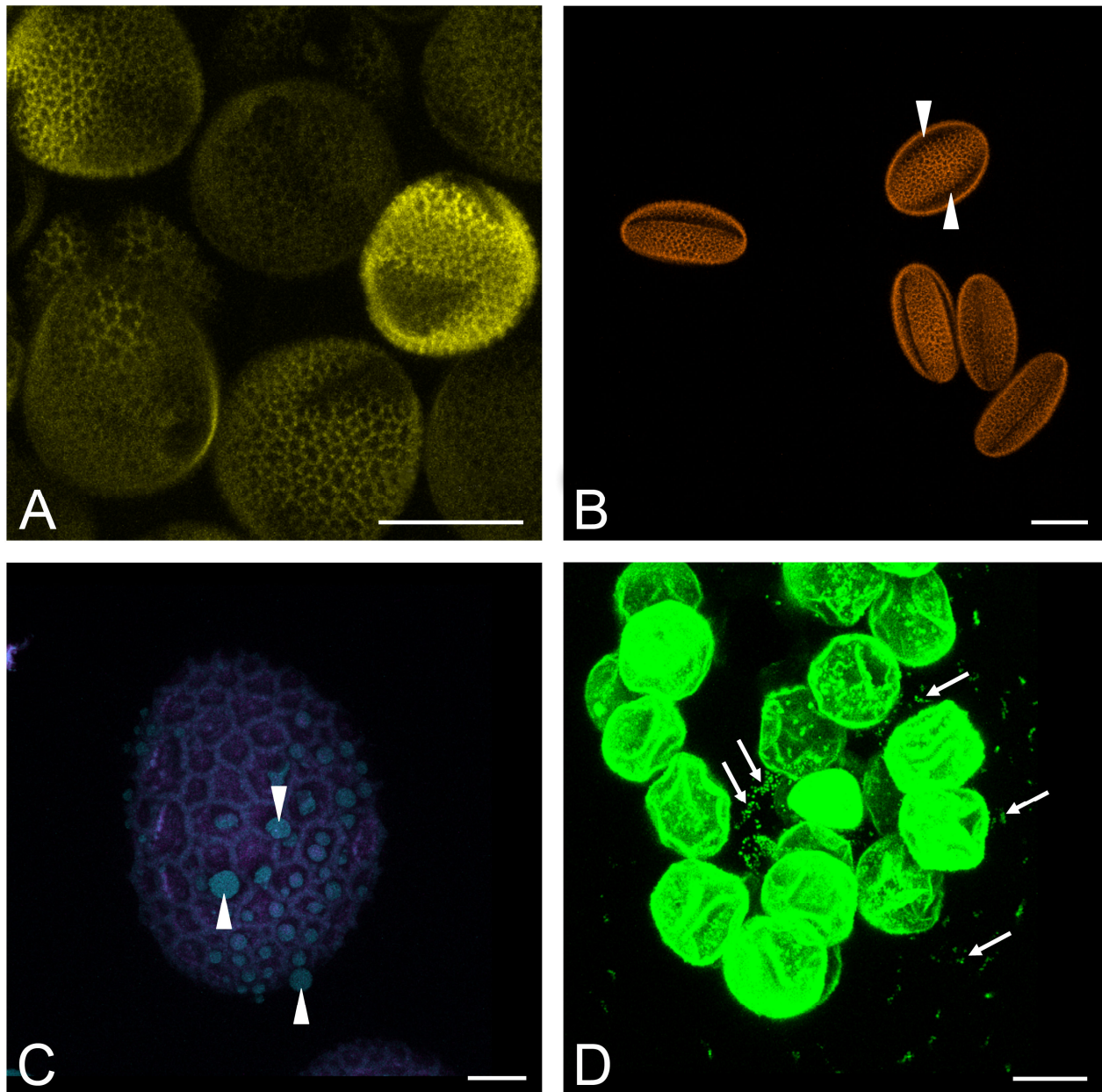
**Table 1** Morphological parameters examined in four plant species using the autofluorescence properties of pollen.

Species	Pollen diameter and shape (P/E)						Exine pattern	Classification of pollen according to the number and type of apertures
	Dry			Hydrated				
	Polar (P) (μm)	Equatorial (E) (μm)	P/E	Polar (P) (μm)	Equatorial (E) (μm)	P/E		
Olive	38.58±6.34	28.14±3.77	1.37	34.37±6.19	31.96±4.20	1.08	Reticulate	Trizonocolporate
Cypress	40.50±9.40	33.64±6.46	1.20	48.00±13.62	44.81±8.12	1.07	Smooth	Inaperturate
Lily	227.34±11.75	86.97±4.74	2.61	194.47±9.22	152.88±7.28	1.27	Reticulate	Monocolpate
Turnip	63.53±4.88	27.05±2.63	2.35	60.53±5.43	40.57±2.51	1.49	Reticulate	Dicolpate

In addition to these morphological traits, imaging of autofluorescence at higher magnification allowed analyzing other characteristics of pollen. In this way, the hydration status of pollen could be easily monitored. Fully hydrated pollen was discernible from partially dehydrated pollen owing to autofluorescence emission was significantly quenched by water. This technique also permitted to detect the presence of additional secreted components over the surface of the pollen wall. We could observe drops of fluorescent material filling the cavities of the exine in the lily pollen (Fig. 3C).



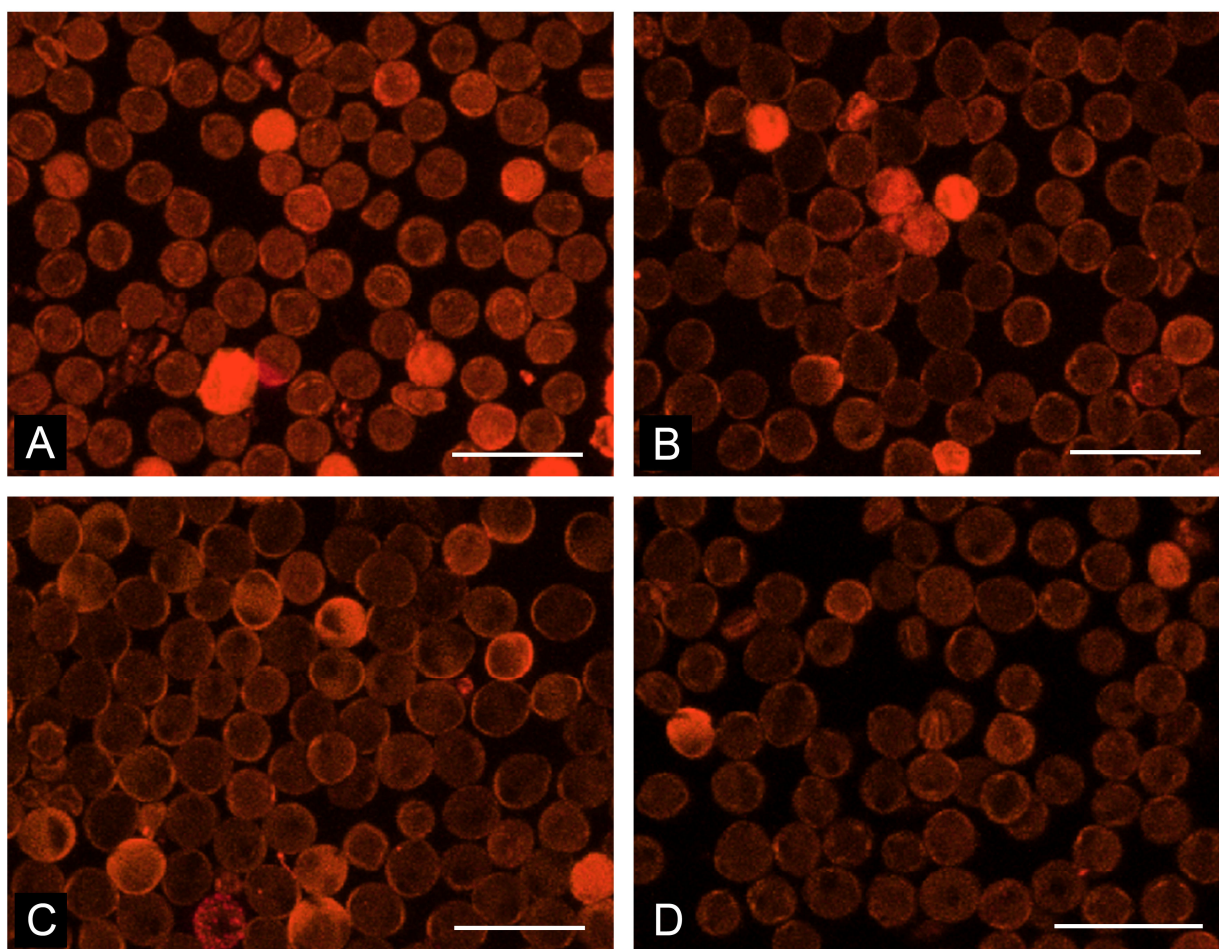
This material, likely of lipidic nature constitutes the “pollen coat”, which is synthesized by the *tapetum* and released to the anther locus after degradation of tapetal cells [24]. Moreover, the material released from pollen to the aqueous medium was also visualized as fluorescent spots scattered in the vicinity of pollen grains (Fig. 3D).



**Fig. 3** (A) Olive pollen showing details of the sculpted exine. (B) Turnip pollen showing the non-fluorescent apertures (arrowheads) in contrast with the fluorescent exine. (C) Lily pollen showing the pollen coat material filling the cavities of the exine (arrowheads). (D) Mediterranean cypress (*Cupressus sempervirens*) pollen showing the presence of fluorescent particles likely originated after disruption of pollen grains and/or secretion into the aqueous medium (arrows). Bars= 20  $\mu$ m.

### 3.2 Olive cultivars identification by pollen autofluorescence

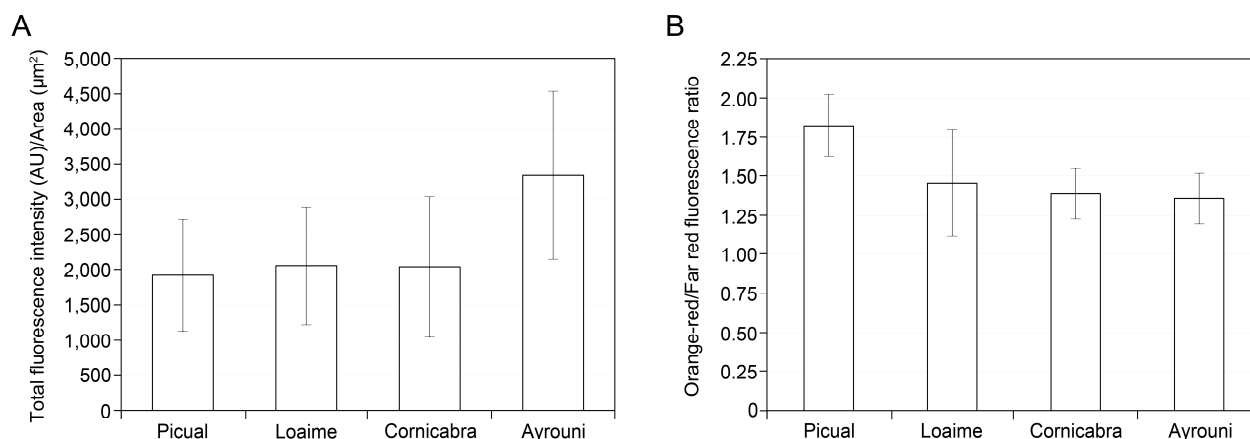
The olive world germplasm is quite broad as a result of its cultivation during millennia [25]. Olive classification into cultivars has been traditionally based on morphological and biometric traits of vegetative organs, as well as on agronomical characteristics [26]. However, reproductive traits have never been considered as a tool for olive identification. Here, we have explored for the first time the prospect of using pollen autofluorescence properties as a tool for varietal discrimination in this species of agronomic interest.



**Fig. 4** Images of autofluorescent olive pollen from cultivars Ayrouni (A), Cornicabra (B), Loaime (C) and Picual (D) after rehydration, showing slight differences in size and autofluorescence intensity. Bars= 100  $\mu$ m.

The autofluorescence emission pattern after pollen rehydration was similar for all cultivars to that described above (Fig. 1E), with some pollen grains being brighter than others (Fig. 4). Similarly, this intra-varietal variability could reflect differences in the water content of each grain within a cultivar. As shown in Fig. 5A, the total autofluorescence intensity significantly varied among cultivars (Kruskal-Wallis test,  $\chi^2 = 28.228$ ,  $p = 0.000$ ). Comparisons using the Mann-Whitney test revealed that Ayrouni presented higher autofluorescence values than Picual, Loaime and Cornicabra cultivars. These differences are likely due to intrinsic factors of pollen (i.e. composition of exine and the material adhered to it) since all cultivars showed similar performance in terms of viability (data not shown).

In all cultivars studied, olive pollen showed a higher fluorescence emission in the range 590-650 nm, although also exhibited significant fluorescence at 650LP nm (Fig. 5B). Interestingly, the orange-red/far red autofluorescence ratio also varied significantly among cultivars (Kruskal-Wallis test,  $\chi^2 = 35.779$ ,  $p = 0.000$ ). Non-parametric comparisons of the autofluorescence ratio for two unpaired groups of species always resulted in significant differences except when Cornicabra and Ayrouni cultivars were compared. Therefore, all together, these parameters allowed classifying the four olive cultivars studied. However, to validate this method for cultivar identification purposes, this study needs to be extended to a larger number of olive cultivars. Moreover, the use of microscopes fitted with spectral detection will help to obtain further data regarding the autofluorescence properties of these samples, and will improve taxonomical discrimination ability.



**Fig. 5** Quantification of total autofluorescence intensity (A) and orange-red/far red autofluorescence ratio (B) of hydrated pollen from four olive cultivars. Means (N= 20)  $\pm$ SD are displayed for each plant species. AU= arbitrary unit.

## 4. Conclusions

Autofluorescence imaging can be considered a non-disruptive method for pollen classification since it does not require any treatment of fixing or staining of the specimens. Therefore, this simple and fast method can be used for pollen counting and taxonomical classification in multiple applications that concern disciplines like Aerobiology, Plant Taxonomy and Cell and Molecular Biology. Moreover, autofluorescence properties of pollen could be used in combination with other morphological parameters to classify olive cultivars.

**Acknowledgements** The support by the Spanish Ministry of Science and Innovation (ERDF-cofinanced project BFU2008-00629) and CSIC (cooperation agreement 2007PT0039) is gratefully acknowledged.

## References

- [1] Chance B, Cohen P, Jobsis F, Schoener B. Intracellular oxidation-reduction states *in vivo*. The microfluorometry of pyridine nucleotide gives continuous measurement of the oxidation state. *Science*. 1962;137:499-508.
- [2] Benson RC, Meyer RA, Zaruba MW, McKhann GM. Cellular autofluorescence – is it due to flavins. *J. Histochem. Cytochem.* 1979;27:44-48.
- [3] Krause GH, Weis E. Chlorophyll fluorescence and photosynthesis: the basics. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 1991;42:313-349.
- [4] Ruhland W, Wetzel K. Der nachweis von chloroplasten in den generativen zellen von pollenschläuchen. *Ber. Deutsch. Bot. Ges.* 1924;42:3-14.
- [5] Berger F. Das verhalten der heufieber-erregenden pollen in filtrien ultravioleten licht. *Beitr. Biol. Pflanzen*. 1934;22:1-12.
- [6] Asbeck F. Fluoreszieren der blütenstaub. *Naturwiss.* 1955;4:632.
- [7] Roshchina VV, Mel'nikova EV, Mit'kovskaya LI, Karnaukhov VN. Microspectrofluorometry for the study of intact plant secretory cells. *Zh. Obshch. Biol.* 1998;59:531-554.
- [8] Roshchina VV, Karnaukhov VN. Changes in pollen autofluorescence induced by ozone. *Biol. Plant.* 1999;42:273-278.
- [9] Roshchina VV, Mel'nikova EV. Pollen chemosensitivity to ozone and peroxides. *Russ. J. Plant Physiol.* 2001;48:74-83.
- [10] Weinbaum SA, Polito VS. Assessment of pollen retention using exine autofluorescence. *Mikroskopie*. 1985;42:278-280.
- [11] Willemse MTM. Changes in the autofluorescence of the pollen wall during microsporogenesis and chemical treatments. *Acta Bot. Neerl.* 1972;21:1-16.
- [12] Willemse MTM. Autofluorescence of pollen wall of *Lilium* and changes in pollen wall of *Gasteria* and *Lilium* anther. *Acta Soc. Bot. Pol.* 1981;50:103-110.
- [13] Audran JC, Willemse MTM. Wall development and its autofluorescence of sterile and fertile *Vicia faba* L. pollen. *Protoplasma*. 1982;110:106-111.
- [14] Suzuzi M, Tonouchi M, Muiyarama K, Mitsumoto K. An analysis of high concentration of pollen at early morning in 2005 spring and a measurement of Japanese cedar and cypress pollen using autofluorescence characteristics. *J. Aerosol Res.* 2005;20:281-289.
- [15] Fonseca AE, Westgate ME, Doyle RT. Application of fluorescence microscopy and image analysis for quantifying dynamics of maize pollen shed. *Crop Sci.* 2002;42:2201-2206.
- [16] Roshchina VV, Mel'nikova EV, Kovaleva LV. Changes in fluorescence during development of the male gametophyte. *Russ. J. Plant Physiol.* 1997;44:45-53.
- [17] Driessen MNBM, Willemse MTM, van Luijin JAG. Grass pollen grain determination by light- and UV- microscopy. *Grana*. 1989;28:115-122.

- [18] Mitumoto K, Yabusaki K, Aoyagi H. Classification of pollen species using autofluorescence image analysis. *J. Biosci. Bioeng.* 2009;107:90-94.
- [19] Stanley RG, Linskens HF, eds. *Pollen: Biology, Biochemistry, Management*. Berlin: Springer-Verlag; 1974.
- [20] Roshchina VV, Popov VI, Novoselov VI, Mel'nikova EV, Peshenko IV, Khutsyan SS, Fesenko EE. Transduction of chemosignal in pollen. *Cytology*. 1998;40:964-971.
- [21] Roshchina VV, Mel'nikova EV, Gordon Rya, Konovalov DA, Kuzin AM. A study of the radioprotective activity of proazulenes using a chemosensory model of *Hippeastrum hybridum* pollen. *Doklady Biophys.* 1998;358:20-23.
- [22] Roshchina VV, Mel'nikova EV, Kovaleva LV. Autofluorescence in the pollen-pistil system of *Hippeastrum hybridum*. *Doklady Biol. Sci.* 1996;349:118-120.
- [23] Mel'nikova EV, Roshchina VV, Karnaukhov VN. Microspectrofluorometry of pollen. *Biophys.* 1997;42:226-233.
- [24] Pacini E, Juniper BE. The ultrastructure of pollen-grain development in the olive (*Olea europaea*). 2. Secretion by the tapetal cells. *New Phytol.* 1979;83:165-174.
- [25] Zohary D, Spiegel-Roy P. Beginnings of fruit growing in the Old World. *Science*. 1975;187:319-327.
- [26] Trujillo I, Morales A, Belaj A, Valpuesta V, Botella MA, Rallo P, Martín A, Dorado G. Identificación de variedades de olivo por marcadores moleculares. In: Rallo L, Barranco D, Caballero JM, Del Rio C, Martín A, Tous J, Trujillo I, eds. *Variedades de olivo en España*. Madrid: Junta de Andalucía, MAPA and Ediciones Mundi-Prensa; 2005:431-450.